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# (54) Anti-cancer compositions containing bacterial extracts

(57) A pharmaceutical composition for treatment of cancerous tumors comprises a dialysis-purified pyridine-soluble extract of a microorganism, together with a refined detoxified endotoxin from one or more Enterobacteriaciae genera, and a pharmaceutically acceptable carrier. The pyridine extract is characterised by a content of about 7–20% by weight of protein, 10–16% by weight of a sugar, and 35–55% by weight of fatty acids. The endotoxin is a material containing 375–475 n.moles per mg. of phosphorus, 1700–2000 n.moles per mg. of fatty acids, and having no detecable 2-keto-3-deoxyocanoate.

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SPECIFICATION

**Pyridine-soluble extract–refined detoxified endotoxin composition and use**

The present invention is directed to a pharmaceutical composition containing refined detoxified endotoxin (RDE) in combination with a pyridine-soluble extract of a microorganism (PE). The RDE used in the present composition is characterized as having no detectable 2-keto-3-deoxyoctanoate, between about 350 and 475 nmoles/mg of phosphorus and between about 1700 and 2000 nmoles/mg of fatty acids. The PE contains between about 3 and 20% by weight of protein, about 10 to 40% by weight of sugar, and about 35 to 60% by weight of fatty acids. The composition is effective to obtain remission and/or regression of cancerous tumors in warm-blooded animals.

Bacteria such as *Corynebacterium parvum* have been the subject of experimental work to isolate and characterize the component responsible for inducing inhibition of tumor growth [see for example, *Anti Tumor Activity and Lymphoreticular Stimulation Properties of Fractions Isolated from C, parvum;* Cantrell, et al, Cancer Research 39, pgs. 3554–3563 (September, 1979)]. Apart from anti-tumor activity, *C. parvum* is a potent stimulator of the lymphoreticular system resulting in undesirable increases in spleen and liver weights and blastogenesis. It has been discovered that a pyridine-soluble extract of a microorganism such as *C. parvum* possesses potent anti-tumor properties without the undesirable toxic effects associated with the prior art products.

Endotoxic extracts obtained from Enterobacteriaciae including parent organisms and mutants are known. These extracts have been used for immunotherapy of various immunogenic tumors [see, *Peptides as Requirement for Immunotherapy of the Guinea-Pig Line-10 Tumor with Endotoxins;* Ribi, et al, Cancer Immunol. Immunother. Vol. 7, pgs. 43–58 (1979)]. However, the endotoxin extracts are known to be highly toxic and, therefore, of limited use in the treatment of cancerous tumors. Efforts have been made to "detoxify" the endotoxins while retaining its tumor regressive capacity. As shown in Ribi, et al, chemical procedures known to detoxify endotoxins while retaining adjuvanticity, such as succinylation and phthalylation resulted in both loss of endotoxicity and tumor regressive potency. Therefore, prior art attempts to obtain an endotoxin product having high tumor regressive potency and little or no toxicity have thus far not been successful.

It is, therefore, an object of the present invention to provide a pharmaceutical composition containing a pyridine-soluble extract of a micro-organism in combination with a refined detoxified endotoxin.

It is another object of the invention to provide a method of treating tumors in warm blooded animals and humans using the composition containing the pyridine-soluble extract of a microorganism and the refined detoxified endotoxin.

*The Pyridine-soluble Extract of a Microorganism (PE)*

The PE contains between about 3 and 20% by weight of protein, about 10 to 40% by weight of sugar, and about 35 to 60% by weight of fatty acids in combination with *C. parvum* whole cells, and preferably contains about 5% by weight of protein, about 35% by weight of sugar and about 55% by weight of fatty acids.

As used herein, there is no limitation with respect to the use of a sugar; all sugars can be used. The same holds true with respect to fatty acids; there is no limitation with respect to the fatty acids which can be used.

The protein comprises amino acids and ammonia and the amino acids include, for example, the following; there was utilized in these determinations, a Beckman amino acids analyzer:

| | |
|---|---|
| Asparginine | 0.273 |
| Threonine | 0.108 |
| Serine | 0.585 |
| Muramic acid | 0.219 |
| Glutamic acid | 0.267 |
| Glycine | 0.39 |
| Alanine | 0.173 |
| Diamino pimelic acid | 0.444 |
| Isoleucine | 0.121 |
| Leucine | 0.167 |
| Phenylalanine | 0.034 |
| Histadine | 0.088 |
| Lysine | 0.544 and |
| Ammonia | 0.524 |

The amounts expressed above are in terms of weight percent and the total protein is 6.34% by weight.

Any microorganism may be used to obtain the pyridine-soluble extract including, for example, *M. bovis* BCG, *M. phlei, M. smegmatis, M. kansasii, Nocardia rubra, Nocardia asteroides, Proprionibacterium acnes* Type II, and *Corynebacterium parvum. Corynebacterium parvum* and *Propionibacterium acnes* Type II are especially preferred.

Whole cells of the microorganism, preferably in the form of a paste, are mixed with pyridine. The resulting mixture is separated to obtain a supernatant fraction which contains the pyridine-soluble extract and a pyridine residue. Optionally, the pyridine residue may be subjected to repeated separation procedures as described above using pyridine to remove further quantities of the desired extract.

The pyridine is then removed from the extract and the dried extract is dialyzed against a suitable liquid such as distilled water. The absence of whole cells or cell fragment contaminants is confirmed by electron microscopy. The resulting purified extract may then be lyophilized by known methods to obtain a stable product.

The pyridine-soluble extract produced in accordance with this invention is combined with RDE to produce a composition having potent anti-tumor activity without stimulating the induction of spleen and liver enlargements. If the pyridine soluble extract is suspended in water, the suspension can be separated into an aqueous soluble and an aqueous insoluble fraction. The aqueous soluble extract is most desirable since it can be easily injected parenterally while at the same time retaining the anti-tumor activity of the pyridine extract. The tumors which may be treated by this composition include animal tumors such as bovine squamous cell carcinoma, bovine fibrosarcoma, equine sarcoid, equine melanona, equine squamous cell carcinoma, canine mammary tumors, canine adenoma and canine melanoma and human tumors such as breast tumors, lung tumors, colon tumors, malignant melanoma, squamous cell carcinomas, ovarian tumors, uterine tumors, bladder and head and neck tumors.

*The Refined Detoxified Endotoxin (RDE)*

Endotoxin extracts of the type used as a starting material to produce RDE may be obtained from any Enterobacteriaciae including parent organisms and mutants. By way of example, the following genera are illustrative of the type of microorganism that may be used: *Salmonella, Shigella, Escherichia, Brucella, Bordetella, Citrobacter, Pseudomonas, Pasturella, Neisseria, Proteus, Klebsiella,* and *Serratia.*

The following species are typically employed: *S. minnesota, S. typhimurium, B. pertussis, B. abortus, S. enteritidis, E. coli, S. typhi, S. marcescens, S. typhosa, Shigella flexni,* and *S. abortus equi.*

The endotoxic extracts used as a starting material may be prepared by one of several known methods [see, for example,

1) Webster, M.E., Sagin, J.F., Landy, M., and Johnson, A.G., *J. Immunol.* 1955, *744,* 55.

2) Westphal, O., Luderitz, O., and Bister, F., *Z. Naturforsch, 76* 148 (1952).

3) Westphal, O., Pyrogens, *Polysaccharides in Biology, Tr. Second Macy Conference* (George F. Springer, ed.). Madison, N.J. Madison Printing Co., 1957, 115.

4) Galanos, C., Luderitz, O., Westphal, O., *Eur. J. Biochem, 9* 245 (1969)

5) Chen, C.H., Johnson, A.G., Kasai, N., Key, B.A., Levin, J., Nowotny, A., *J. Infect. Dis. 128* 543 (1973).

6) Ribi, E., Haskins, W.T., Landy, M., Milner, K.C., The *Journal of Experimental Medicine 114* 647 (1961).

7) Leive, L., *Biochem. Biophys. Res. Comm. 21* 290 (1965).

8) Ribi, E., Milner, K.C., and Perrine, T., *J. Immunol. 82* 75 (1959].

The preferred method of obtaining the endotoxic extract is that disclosed by Chen et al; namely, methanolchloroform precipitation.

The methanol-chloroform precipitate (MCP) is then reacted with an organic or inorganic acid and then lyophilized to produce a hydrolyzed crude lipid A with reduced toxicity and pyrogenicity as compared with the starting endotoxin material. This material is then treated with a solvent which is capable of specifically dissolving fatty acids and other impurities without dissolving the crude lipid A. The phosphate content of the detoxified, refined lipid A is about one-half that observed for toxid endotoxin suggesting that the phosphate content is related to the toxic effects of endotoxins.

The preferred inorganic acids used to react with MCP are hydrochloric acid, sulfuric acid, or phosphoric acid and the preferred organic acids are toluene sulphonic acid or trichloroacetic acid. The reaction may suitably be conducted at a temperature between about 90° and 130°C, for a time sufficient to complete hydrolysis, usually between about 15 and 60 minutes.

The preparation of crude detoxified endotoxin may be accomplished by reacting the starting material with the acid in the presence of an organic solvent such as chloroform, methanol, and ethanol or combinations thereof.

The resulting crude lipid A is suspended in acetone which is the preferred solvent for dissolving the fatty acids and other impurities. The solvent is then removed to produce crude detoxified endotoxin.

The crude detoxified endotoxin is then dissolved in a solvent and the solution is passed through a suitable chromatographic column such as a molecular exclusion chromatographic column, to separate the RDE fractions which are then combined afer removal of the solvent. The crude detoxified endotoxin solution is passed through a Sephadex column in the presence of a solvent such as chloroform, methanol, acetone, pyridine, ether or acetic acid or combinations thereof. The pressure of the column may vary but is typically in the range of between about atmospheric and 100 lbs/in$^2$ and the flow rate is between about 0.1 and 10 ml/min.

The crude detoxified endotoxin solution may be passed through a DEAE-cellulose column under the same pressure conditions as mentioned above for the Sephadex column. The flow rate is maintained between about 2 and 15 ml/min. The solvents used are also the same as used for the Sephadex column although water and/or diethylamine can be added to all mixtures at a concentration of up to about 1%.

Other methods of producing RDE from crude detoxified endotoxin include passing the solution through a low pressure silica-gel 60 column having a particle size of between about 25 and 63 microns and using a solvent comprised of chloroform, methanol, water and ammonium hydroxide. The preferred volume ratio of the components of the solvent is about 50:25:4:2.

The refined detoxified endotoxin (RDE) has no detectable 2-keto-3-deoxyoctanoate, between about 350 and 475 nmoles/mg of phosphorus and between about 1700 and 2000 nmoles/mg of fatty acid.

The composition is administered by injection in a pharmaceutically acceptable medium such as an oil-droplet emulsion or a physiologic saline solution, and is preferably administered directly into the tumor under conditions more particularly described below. Administration can be by IV injection, or by IV infusion.

The composition may be stabilized, as, for example, by a lyophilization procedure and then reconstituted without loss of potency.

The amount of RDE in a single injection for treatment of animals is between about 25–500 micrograms/ml, suitably between 50 and 100 micrograms/ml. and the amount of PE is between about 25–500 and suitably between about 100 and 250 micrograms/ml.

The number of milliliters of the biologic injected into the tumor is determined by the size of the tumor in accordance with the following table:

*Animal Dosage According to Tumor Size*

| Diameter of Tumor (cm) | Amount of Biologic Injected (ml) |
|---|---|
| 0–1 | up to 0.5 |
| 1–2 | 0.5 to 2.5 |
| 2–3 | 2.5 to 5 |
| 3–5 | 5 to 10 |
| 5–8 | 10 to 15 |
| greater than 8 | 15 to 20 |

The maximum dose per injection is about 10 milligrams of RDE and about 25 of PE. The course of treatment comprises up to 4 to 10 injections administered at about two week intervals.

The present composition in a suitable injection medium such as physiologic saline solution is administered directly into human tumors. The amount of RDE in a single injection is between about 5 and 1,000 micrograms, suitably between about 25 and 500 micrograms. The amount of PE is between about 50 and 5,000 micrograms, suitably between about 200 and 3000 micrograms. The preferred dosage level for RDE is about 100 micrograms and for PE it is about 1000 micrograms. All of the above-mentioned dosage levels are based on a typical 70 kilogram adult patient. The injections are administered about once every week for up to a total of about 15 injections.

As mentioned above the composition for treatment of warm blooded animals and humans may be used in the form of a saline or an oil droplet emulsion. The amount of oil used is in the range of between about 0.5 and 3.0 percent by volume based on the total volume of the composition. It is preferred to use between about 0.75 and 1.5 percent by volume of the oil. Examples of such oils include light mineral oil, squalane, 7-n-hexyloctadecane, Conoco superoil and Drakeol 6 VR mineral oil (produced by the Pennreco Company, Butler, Pennsylvania).

The homogenized oil containing mixture is then combined with a detergent which may optionally be dissolved in a saline solution prior to mixing. The amount of detergent is typically between about 0.02 and 0.20 percent by volume and preferably between about 0.10 and 0.20 percent by volume based on the total volume of the composition. Any common detergent material may be used including Tween-80, and Arlacel (produced by the Atlas Chemical Company).

The mixture resulting from the addition of detergent is then homogenized to form a suspension which has a high percentage of oil droplets coated with the active components as determined by observation under a microscope.

The following examples are for illustrative purposes only and are not intended to limit or in any way redefine the invention as claimed in the claims appended hereto.

*Example 1*—Preparation of Pyridine-Soluble Extract from *Proprionibacterium acnes* Type II (Strain VPI 0204)

*Propionibacterium acnes* Type II (Strain VPI 0204) was grown and harvested at 37°C. in NIH thioglycolate broth for between 48 and 72 hours to obtain a whole cell paste. The paste was then washed with 500 ml of distilled water. 90 grams (wet weight) of the washed paste was mixed with 200 ml. of neat pyridine and centrifuged at 1700 × g for one hour at 4°C. A pyridine-soluble extract was removed as a supernatant fraction. The remaining residue was extracted with additional pyridine under identical conditions as described above. Following filtration, using Whatman No. 1 Paper, the pyridine extracts were pooled and the solvent was removed by evaporation at 50°C in a Buchi Rotavapor (Brinkmann Instruments, Westbury, New York). The dried pyridine extract was extensively dialyzed against distilled water and then lyophilized. The resulting purified pyridine extract contained about 5% by weight of protein, about 35% by weight of sugar and about 55% by weight of fatty acids. The extract was examined under an electron microscope and found to be free of contaminating whole cells and cell wall fragments. The yield of the pyridine-soluble extract was 9% (8.1g.).

*Example 2*—Preparation of Pyridine-Soluble Extract *M. bovis* Strain BCG

*M. bovis* strain BCG was grown and harvested in Sautons medium at 37°C for between 3–4 weeks to obtain a washed whole cell paste. 50 grams (wet weight) of the washed paste was then treated in the same manner as Example 1 to produce a yield of the pyridine-soluble extract of 7% (3.5g). The extract contained 15% by weight of protein, 10% by weight of sugar and 52% by weight of fatty acids.

*Example 3*—Preparation of Aqueous Extract

500 mg of pyridine extract was sonicated in 100 ml. of distilled water for 15–30 minutes. The resulting suspension was centrifuged at 12,000 rpm in an RC2B centrifuge at 4°C for 40 minutes. The supernatant was decanted and saved. The residue was extracted two more times, as above. The supernatants were combined in a lyophilizing bottle, shell frozen and lyophilized. Yield 230 mg (46%).

*Example 4*—Preparation of Crude Detoxified Endotoxin

A 650 mg sample of a methanol-chloroform precipitate produced in accordance with the procedure of Chen, et al *J. Infect. Dis. 128 543 (1973)* was suspended in 150 ml of 0.1 N HCl. in a three necked round bottom flask fitted with a condenser, and immersed in a sonicator. After sonication, the glass apparatus was then lowered into an oil bath maintained at 120°C. which allowed the inerior temperature of the flask to approach or exceed the boiling point of the solution. Superheating of the solution was minimized by fitting the flask with a capillary tube attached to a nitrogen gas source through one of the necks. A continuous flow of nitrogen was maintained throughout the hydrolysis procedure.

Hydrolysis was continued for 30 minutes, and then the solution was cooled in an ice bath, sonicated to disperse the solid material and distributed in corex tubes. The flask was washed with distilled water to remove all solid material adhering to the sides of the flask, and the wash was added to the suspension in the corex tubes. Centrifugation was carried out at 12,000 rpm for 80 minutes. The supernatant was decanted and discarded. The solid residue was resuspended in distilled water, sonicated until the suspension was well dispersed and re-centrifuged. The centrifugation process was then repeated. The residue was taken up in distilled water, shell frozen and lyophilized yielding 382 mg of crude lipid A. 150 mg of this material was treated with cold (0°C) acetone to remove fatty acids, sonicated, and filtered through a Whatman No. 1 gravity filtration apparatus at 5°C. 100 mg of crude detoxified endotoxin remained after drying.

*Example 5*—Preparation of Crude Detoxified Endotoxin

A 120 mg sample of MCP (methanol-chloroform precipitate) was suspended in 12 ml of absolute methanol, sonicated to disperse solid materials and distributed into 6 (1 × 10 cm)

screw cap vials. 2 ml of 0.2N HCl were added to each tube and the resulting suspension was incubated in a boiling water bath for 45 minutes. After hydrolysis, the tubes were cooled in an ice water bath and centrifuged for about 10 minutes at 2500 rpm. The supernatant was decanted and 5 ml of a 2:1 chloroform/methanol mixture were added to the residue to effect dissolution. 2 ml of water were added per tube and the solution was mixed. The biphasic solution was recentrifuged at 2500 rpm for 10 minutes. The upper water phase was discarded and 1 ml of a 4:1 chloroform/methanol mixture was added to each tube resulting in a clear solution. The solutions were pooled, and the solvent evaporated on a rotary evaporator. The residue was dried under high vacuum and lyophilized to yield 45 mg of crude lipid A. 20 mg of this material were treated with cold (0°C) acetone, sonicated, and filtered through a Whatman No. 1 gravity filtration apparatus at 5°C. 13 mg of crude detoxified endotoxin remained after drying.

*Example 6*–Preparation of Refined Detoxified Endotoxin

110 g LH-20-100 (25–100 micron particle size: Pharmacia) were combined with 600 ml of a 2:1 chloroform/methanol mixture which was permitted to stand for 30 minutes. The resulting slurry was added to a 25 × 1000 mm glass chromatography column (BRL Laboratories) fitted with pressure fittings. After packing was completed, the column was attached by means of Teflon pressure tubing to an ISCO Model 132 pump. 400 ml of a 4:1 chloroform/methanol mixture were pumped through the column at the rate of 3 ml/min. 100 mg of crude detoxified endotoxin prepared in accordance with Example 4 were applied to the column in 2.5 ml of a 4:1 chloroform/methanol mixture via a sample loop. The flow was reduced to 1 ml/min. and after 150 ml of eluant were collected, the effluent was connected to a fraction collector. 4 ml fractions were collected and refined detoxified endotoxin fractions were determined by thin layer chromatographic analysis of the fractions [E. Merck, 0.25 mm thick, chloroform/methanol/$H_2O$/$NH_4OH$ (50:25:4:2) as eluant].

The refined detoxified endotoxin fractions were combined and the solvent evaporated leaving 30 mg of refined detoxified endotoxin as a white powder.

*Example 7*–Preparation of Refined Detoxified Endotoxin

33g of DEAE-cellulose (Whatman DE–32) were suspended in 150 ml of glacial acetic acid and agitated gently for 10 minutes to obtain a slurry powder. The mixture was set aside overnight.

The slurry was poured into a 25 × 400 mm column, allowed to settle with tapping, and excess acid was thereafter drained. The column was washed with 2000 ml of methanol followed by 200 ml of a 4:1 chloroform/methanol mixture. A 100 mg sample of crude detoxified endotoxin produced in accordance with Example 4 was added to the column in 3 ml of a 4:1 chloroform/methanol mixture or an 80:20:1 mixture of chloroform, methanol and water. The column was eluted with 350 ml of a 4:1 chloroform/methanol mixture followed by 300 ml of a 99:1 methanol/water mixture. Using a linear gradient apparatus, the column was eluted with 2000 ml of a linear gradient starting with 100% methanol and ending with 0.2 M acetic acid in methanol. The column was eluted at the rate of 6 ml/min. and 15 ml fractions were collected. Every other fraction was analyzed for total phosphorous content according to the procedure of Bartlett, G.R., *J. Biol. Chem. 234*, 466–471 (1959). The fractions were pooled and evaporated on a rotary evaporator to near dryness and taken up in 10 ml of a 2:1 chloroform/methanol mixture and 40 ml of 0.001 M acetic acid in a separatory funnel. The lower layer was separated, filtered through Whatman No. 2 filter paper and evaporated to dryness to yield 19.2 mg of refined detoxified endotoxin.

*Example 8*

23 eight to ten week old female C3HeBFeJ mice were injected intraperitoneally with $10^5$ ovarian teratocarcinoma cells. After 24 hours, five mice were injected once with 0.2–0.5 ml of an isotonic saline solution containing 50 micrograms of RDE and six mice were injected once with 0.2–0.5 ml of the saline solution containing 300 micrograms of PE and 50 micrograms of RDE. Finally, twelve mice were injected once with 0.2–0.5 ml of the saline solution as a control. After 21 days, 4 of the 5 mice injected with RDE showed complete regression of the tumor and 6 of 6 of the mice injected with RDE and PE showed similar results. On the other hand, 10 of 12 mice of the control group died by the twenty-first day and the remaining two still showed evidence of the cancer cells.

*Example 9*

Forty-five 8–10 week old female C3HEJ mice were injected with $10^5$ ovarian teratocarcinoma. After 24 hours, 15 of the mice were injected once with 0.2–0.5 ml of an isotonic saline solution containing 1400 micrograms of PE and 15 of the mice were injected once with 0.2–0.5 ml of the saline solution containing 300 micrograms of PE and 50 micrograms of RDE.

Finally, 15 mice were injected once with 0.2–0.5 ml of the saline solution as a control. After 30 days, 5 of the 15 mice injected with PE were still living and 8 of the 15 mice injected with RDE and PE had shown tumor regression and were still living. On the other hand, 14 of the 15 mice of the control group had died with the remaining 1 mouse showing tumor regression.

CLAIMS

1. A composition comprising a therapeutically effective amount of:

(a) a purified pyridine-soluble extract obtained from a microorganism said extract containing between about 7 and 20% by weight of protein, between about 10 and 16% by weight of sugar, and between about 35 and 55% by weight of fatty acids.

(b) refined detoxified endotoxin having no detectable 2-keto-3-deoxyoctanoate and having between 375 and 475 nmoles/mg of phosphorus and between about 1700 and 2000 moles/mg of fatty acids; and

(c) a pharmaceutically acceptable carrier.

2. The composition of claim 1, wherein the microorganism is *M. bovis* BCG, *M. phlei, M. smegmatis, M. kansasii, Nocardia rubra, Nocardia asteroides, Proprionibacterium acnes* Type II, and *Corynebacterium parvum.*

3. The composition of claim 1 or 2, wherein said microorganism is *Corynebacterium parvum.*

4. The composition of claim 1 or 2, wherein said microorganism is Propionibacterium acnes Type II.

5. The composition according to any of the preceding claims wherein said extract contains about 12% by weight of each of protein and sugar and about 45% by weight of fatty acids.

6. The composition according to any of the preceding claims wherein the ratio of said extract to said refined detoxified endotoxin is from 1:1 to 100:1.

7. The composition according to any of the preceding claims wherein the amount of said extract is between about 50 and 5000 micrograms and the amount of said refined detoxifed endotoxin is between about 5 and 1000 micrograms.

8. The composition of claim 7, wherein the amount of said extract is about 500 micrograms and the amount of said refined detoxified endotoxin is about 100 micrograms.

9. The composition according to any of the preceding claims in lyophilized form.

10. The composition according to any of the preceding claims wherein said carier is physiological saline solution.

11. The composition according to any of claims 1 to 9 in the form of an oil droplet emulsion.

12. The composition of claim 11, wherein said oil is a light mineral oil, squalane, or 7-n-hexyl octadecane.

13. Th composition of claim 11, wherein said oil is present in an amount between about 0.5 and 3.0% by volume based on the total volume of the composition.

14. The composition according to any of the preceding claims further comprising a detergent in an amount between about 0.02 and 0.25% by volume based on the total volume of the composition.

15. A therapeutic composition substantially as described herein.

16. A method of producing an immunological response in a warm blooded animal comprising administering the composition of claims 1 to 15 to said warm blooded animal.

17. The method of claim 16, further comprising injecting said composition parenterally or directly into the tumor tissue for up to 15 injections.

18. The method of claim 16 or 17, wherein said injections are made at intervals of at least one week.

19. A method of producing a immunological response in humans comprising administering the composition of claims 1 to 15 to said human.

20. The method of claim 19, further comprising injecting said composition directly or parenterally into the tumor tissue for up to 15 injections.

21. The method of claim 19 or 20, wherein said injections are made at intervals of one week.

22. A method of producing an immunological response in a warm blooded animal substantially as described herein.

23. A method of treating tumors in warm blooded animals comprising administering the composition of claims 1 to 15 in said warm blooded animals.

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# United States Patent [19]

Cantrell

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[54] **PYRIDINE SOLUBLE EXTRACT OF A MICROORGANISM**


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**Related U.S. Application Data**

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[58] Field of Search ..... 424/195



[56] **References Cited**

U.S. PATENT DOCUMENTS

| | | | |
|---|---|---|---|
| 3,956,481 | 5/1976 | Jollcs et al. | 424/92 |
| 3,976,544 | 8/1976 | Adam et al. | 424/92 |

OTHER PUBLICATIONS

Meyer et al., J. Natl. Can. Inst., 52:103–108, 1974.
Ribi et al., Natl. Can. Inst. Monograph, No. 39, 1974.
Rozenoweig et al., Canc., 40:334–342, 1977.
Pharmacological Basis of Cancer Chemoth., Williams & Wilkins Co., 1975, pp. 245–270.



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[57] **ABSTRACT**

A pharmaceutical composition is disclosed comprising a purified pyridine-soluble extract obtained from a microorganism which contains between about 7 and 20% by weight of protein, between about 10 and 16% by weight of sugar, and between about 35 and 55% by weight of fatty acids which when combined with cell wall skeleton and trehalose dimycolate in a pharmaceutically acceptable medium is useful as an anti-animal tumor agent in the treatment of animals.


**7 Claims, No Drawings**

# PYRIDINE SOLUBLE EXTRACT OF A MICROORGANISM

This application is a continuation of application Ser. No. 393,822, filed June 20, 1982, now abandoned.

## BACKGROUND OF THE INVENTION

The present invention is directed to a pyridine-soluble extract of a microorganism which, when combined with cell wall skeleton (CWS) and trehalose dimycolate (TDM) provides a pharmaceutical composition possessing anti-animal tumor properties.

Bacteria such as *Corynebacterium parvum* have been the subject of experimental work to isolate and characterize the component responsible for inducing inhibition of tumor growth [see, for example, *Anti Tumor Activity and Lymphoreticular Stimulation Properties of Fractions Isolated from C. parvum;* Cantrell, et al., Cancer Research 39, pgs. 3554–3563 (September, 1979)]. Apart from anti-tumor activity, *C. parvum* has shown to be a potent stimulator of the lymphoreticular system resulting in undesirable increases in spleen and liver weights and blastogenesis. Applicant has discovered that a pyridine-soluble extract of a microorganism possesses potent anti-animal tumor properties without the undesirable toxic effects associated with the prior art products.

Cell wall skeleton is essentially cell wall which has had much of the protein and lipids normally found in the cell wall removed. It is a polymeric mycolic acid arabinogalactan mucopeptide containing remnants of trehalose mycolates ("P3") and undigested tuberculoproteins. Cell wall skeleton is obtained from any microorganism including, but not limited to, *M.smegmatis, M.phlei, Nocardia rubra, Nocardia asteroides, Corynebacterium diphtheria, Corynebacterium parvum, M.kansasii, M.tuberculosis* (Strain H 37 and RV and Ayoma B), and M.bovis Strain BCG. Additionally, cell wall skeleton may be obtained from such other organisms as *E.coli, B.abortus* and *Coxiella burnettii.*

Cell wall skeleton may be produced by first growing and harvesting bacteria such as *M.bovis* strain BCG (Bacillus Calmette-Guerin). The resulting whole cell residue is processed through a cell fractionator [Ribi Cell Fractionator (Sorvall, Model RF-1)] which disrupts the cells, separating the outer envelope or cell wall from the protoplasmic impurities. The resulting cell walls are then subjected to a series of solvent extractions and enzymatic treatments (e.g., trypsin and/or chymotrypsin) to give purified cell wall skeleton.

Trehalose dimycolates (TDM), may be obtained from the organisms such as, for example, *M.avium, M.phlei, M.tuberculosis* (Strain H 37 RV and Ayoma B), *M.bovis* BCG, *M.smegmatis, M.kansasii, Nocardia rubra, M.bovinis* and *Corynebacterium diphtheriae.*

Bacteria such as *M.avium* are grown, harvested and then heat killed. The cell mass is then extracted with several solvents and then an active, solvent soluble, fraction is extracted. This extract is further purified by a series of solvent extractions to provide crude TDM (see *Biologically Active Components from Mycobacterial Cell Walls. I. Isolation and Composition of Cell Wall Skeleton and Component p3*; Azuma, et al., Journal of the National Cancer Institute, Volume 52, pgs. 95–101, 1974) incorporated herein by reference. As disclosed in Azuma et al., crude TDM may then be further purified by centrifugal microparticulate silica gel chromatography to give purified TDM.



It is, therefore, an object of the present invention to provide a pharmaceutical composition containing a pyridine-soluble extract of a microorganism in combination with cell wall skeleton and trehalose dimycolate.

It is another object of the invention to provide a method of treating animal tumors in warm blooded animals using the composition containing the pyridine-soluble extract of a microorganism, cell wall skeleton and trehalose dimycolate.

## SUMMARY OF THE INVENTION

The present invention relates to pharmaceutical compositions comprising a pyridine-soluble extract of a microorganism containing about 7 and 20% by weight of protein, about 10 and 16% by weight of sugar and about 35 to 55% by weight of fatty acids in combination with cell wall skeleton (CWS) and trehalose dimycolate (TDM). The extract preferably contains about 12% by weight of each of protein and sugar and about 45% by weight of fatty acids.

Any microorganism may be used to obtain the pyridine-soluble extract including, for example, *M.bovis* BCG, *M. phlei, M. smegmatis, M. kansasii, Nocardia rubra, Corynebacterium diptheriae* and *Corynebacterium parvum. Corynebacterium parvum* is especially preferred.

Whole cells of the microorganism, preferably in the form of a paste, are mixed with pyridine. The resulting mixture is separated to obtain a supernatant fraction which contains the pyridine-soluble extract and a pyridine residue. Optionally, the pyridine residue may be subjected to repeated separation procedures as described above using pyridine to remove further quantities of the desired extract.

The pyridine is then removed from the extract and the dried extract is dialyzed against a suitable liquid such as distilled water. The absence of whole cell and cell fragment contaminants is confirmed by electron microscopy. The resulting purified extract may then be lyophilized by known methods to obtain a stable product.

The pyridine-soluble extract produced in accordance with this invention may be combined with CWS and TDM to produce a composition having potent anti-animal tumor activity without stimulating the induction of spleen and liver enlargements. The cancers which may be treated by this composition include animal tumors such as bovine squamous cell carcinoma, bovine fibrosarcoma, equine sarcoid, equine melanoma, equine squamous cell carcinoma, canine mammary tumors, canine adenoma and canine melanoma.

The composition is preferably administered by injection in a pharmaceutically acceptable medium such as an oil-droplet emulsion directly into the tumor under conditions more particularly described below. The aforesaid composition may be stabilized as for example, by a lyophilization procedure and then reconstituted without loss of potency.

The amount of the pyridine-soluble extract in a single injection for the treatment of animals is between about 375 and 2500 micrograms/milliliter. The amount of each of CWS and TDM is between about 125 and 375 micrograms/milliliter.

The number of milliliters of the biologic injected into the tumor is determined by the size of the tumor in accordance with the following table:

| Animal Dosage According to Tumor Size | |
|---|---|
| Diameter of Tumor (cm) | Amount of Biologic Injected (ml) |
| 0–1 | up to 0.5 |
| 1–2 | 0.5 to 2.5 |
| 2–3 | 2.5 to 5 |
| 3–5 | 5 to 10 |
| 5–8 | 10 to 15 |
| greater than 8 | 15 to 20 |

The maximum dose per injection is about 40 milligrams for the pyridine-soluble extract, 40 milligrams for CWS, and 6 milligrams for TDM. The course of treatment comprises up to six injections administered at about two week intervals.

The present composition in a suitable injection medium such as an oil-droplet emulsion is administered directly into animal tumors. The amount of the pyridine-soluble extract in a single injection is between about 200 and 5000 micrograms, preferably between 800 and 1200 micrograms. The amount of CWS is between about 50 and 2000 micrograms while the amount of TDM is between about 50 and 1000 micrograms. The preferred single dosage level for each of CWS and TDM is between about 475 and 525 micrograms. All of the above-mentioned dosage levels are based on a typical 70 kilogram adult animal. The injections are administered about once every week for up to a total of 15 injections.

As described above, the composition for treatment of warm blooded animals may be used in the form of an oil droplet emulsion. The amount of oil used is in the range of between about 0.5 and 3.0 percent by volume based on the total volume of the composition. It is preferred to use between about 0.75 and 1.5 percent by volume of the oil. Examples of such oils include light mineral oil, squalane, squalane, and 7-n-hexyl-octadecane.

The homogenized oil containing mixture is then combined with a detergent which may optionally be dissolved in a saline solution prior to mixing. The amount of detergent is typically between about 0.2 and 0.25 percent by volume and preferably between about 0.10 and 0.20 percent by volume based on the total volume of the composition. Any common detergent material may be used including Tween-80 and Arlacel (produced by the Atlas Chemical Company).

The mixture resulting from the addition of detergent is then homogenized to form a suspension which has a high percentage of oil droplets coated with the active components as determined by observation under a microscope.

The following examples are set for illustrative purposes only and are not intended to limit or in any way redefine the invention as claimed in the claims appended hereto.

## EXAMPLE 1

### Preparation of Pyridine-Soluble Extract from *Corynebacterium Parvum*

*Corynebacterium parvum* (P.acnes, Strain 4182) was grown and harvested at 37° C. in NIH thioglycolate broth for between 48 and 72 hours to obtain a whole cell paste. The paste was washed with 500 mg. of distilled water. 90 grams (wet weight) of the washed paste was mixed with 200 ml. of neat pyridine and centrifuged at 1700×g for one hour at 4° C. A pyridine-soluble extract was removed as a supernantant fraction. The remaining residue was extracted with additional pyridine under identical conditions as described above. Following filtration, using Whatman No. 1 paper, the pyridine extracts were pooled and the solvent was removed by evaporation at 50° C. in a Buchi Rotavapor (Brinkmann Instruments, Westbury, N.Y.). The dried pyridine extract was extensively dialyzed against distilled water and then lyophilized. The resulting purified pyridine extract contained about 12% by weight of protein, about 12% by weight of sugar and 45% by weight of fatty acids. The extract was examined under an electron microscope and found to be free of contaminating whole cells and cell wall fragments. The yield of the pyridine-soluble extract was 9% (8.1 g.).

## EXAMPLE 2

### Preparation of Pyridine-Soluble Extract from *M.bovis* Strain BCG

*M. bovis* Strain BCG was grown and harvested in Sautons medium at 37° for about 3 to 4 weeks to obtain a washed whole cell paste. 50 grams (wet weight) of the washed paste was then treated in the same manner as Example 1 to produce a yield of the pyridine-soluble extract of 7% (3.5 g). The extract contained 15% by weight of protein, 10% by weight of sugar and 52% by weight of fatty acids.

## EXAMPLE 3

### Guinea-Pig Line-10 Tumor Tests

Six strain 2 guinea pigs having Line-10 tumor growths of about 9 mm. in diameter were injected once with 0.4 ml of a sterile oil droplet emulsion, i.e., Drakeol 6 VR mineral oil (Pennsylvania Refining Company, Butler, Pa.), containing 300 micrograms of the pyridine-soluble extract prepared in accordance with Example 1 and 50 micrograms of each of cell wall skeleton and trehalose dimycolate, directly into the tumor tissue.

At the end of three months, the animals were examined and in 5 of the 6 animals, total regression had occurred.

In a control experiment, six strain 2 guinea pigs having Line-10 tumor growths of about 9 mm. in diameter were injected once with 0.4 ml of the sterile oil droplet emulsion described above without the pyridine extract or cell wall skeleton and trehalose dimycolate. The injections were made directly into the tumor tissue. None of the six tumors showed any signs of regression after three months.

What is claimed is:

1. A pharmaceutical composition for treating tumors selected from the group of bovine squamous cell carcinoma, bovine fibrosarcoma, equine sarcoid, equine melanoma, equine squamous cell carcinoma, canine mammary tumors, canine adenoma and canine melanoma, comprising an anti-tumor effective amount of a combination of an effective amount of a purified pyridine-soluble extract obtained from a microorganism selected from the group consisting of *M. bovis* BCG, *M. phlei, M. smegmatis, M. kansasii, Nocardia rubra, Corynebacterium diphtheriae* and *Corynebacterium parvum*, said extract obtained by:

(a) preparing a whole cell paste of said microorganism;

(b) washing said paste;

(c) treating said paste with pyridine to produce an extract and a residue;

(d) removing said pyridine from said extract; and

(e) dialyzing said dried extract to obtain said purified pyridine-soluble extract, an effective amount of cell wall skeleton; and an effective amount of trehalose dimycolate; and a pharmaceutically acceptable carrier.

2. The composition of claim 1 wherein the amount of each of said pyridine-soluble extract and cell wall skeleton is up to about 40 milligrams and the amount of trehalose dimycolate is up to about 6 milligrams.

3. The composition of claim 1 wherein the composition is in lyophilized form.

4. The composition of claim 1 wherein the composition is in the form of an oil-droplet emulsion.

5. The composition of claim 2 wherein the amount of said pyridine-soluble extract product is between about 200 and about 5000 micrograms, the amount of cell wall skeleton is between about 50 and 2000 micrograms and the amount of trehalose dimycolate is between about 50 and 1000 micrograms.

6. A method of treating tumors selected from the group consisting of bovine squamous cell carcinoma, bovine fibrosarcoma, equine sarcoid, equine melanoma, equine squamous cell carcinoma, canine mammary tumors, canine adenoma and canine melanoma in warm blooded animals comprising administering an effective amount of the composition of claim 1 to said warm blooded animals by injection directly into the tumor.

7. The method of claim 6 further comprising injecting into said tumor said composition containing between about 375 and 2500 micrograms/milliliters of said pyridine-soluble extract product and between about 125 and 375 micrograms/milliliters of each of said cell wall skeleton and trehalose dimycolate.

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